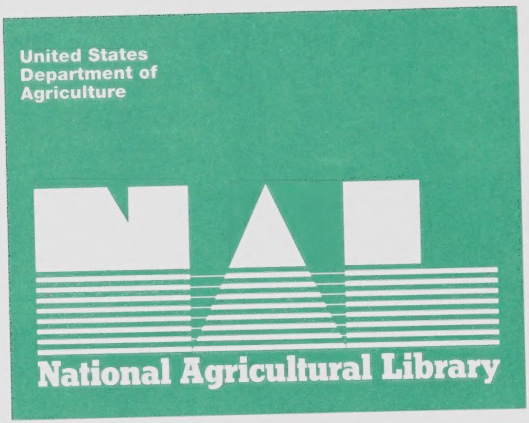
LABORATORY METHODS

in Veterinary Mycobacteriology

VETERINARY SERVICES LABORATORIES
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
U. S. DEPARTMENT OF AGRICULTURE
AMES, IOWA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LABORATORY METHODS

in

Veterinary Mycobacteriology

for

The Isolation and Identification

of

MYCOBACTERIA

Prepared by the Staff

of

The Mycobacteriology Unit

Veterinary Services Laboratories

Animal and Plant Health Inspection Service

United States Department of Agriculture

Ames, Iowa

Revised January 1, 1974

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TABLE OF CONTENTS

Page

HISTORY OF MYCOBACTERIOLOGY UNIT, VETERINARY SERVICES LABORATORIES, NADC

In 1958, a mycobacteriology unit was formed as part of an interim laboratory of the Animal Disease Eradication Division located at the School of Veterinary Medicine, Iowa State University, Ames, Iowa. The original staff consisted of a veterinarian, a microbiologist, and an animal caretaker. They were assigned the responsibility of developing the laboratory capabilities necessary to maintain stock cultures and to isolate mycobacteria from tuberculous animal tissues submitted by veterinary meat inspectors. The limited number of cases processed during the first year started the first flow of urgently needed culture information to the regulatory veterinarians concerned with tuberculous herds in the United States. Mycobacterial sensitizing material was produced for use in an important program designed to familiarize regulatory veterinarians with the application and interpretation of the tuberculin skin test.

The facilities were moved to the National Animal Disease Laboratory in 1961. Investigational projects were developed by unit personnel to solve existing problems and provide better methods and products. A rapid method was adopted in 1973 which markedly reduced the time required for processing specimens for routine mycobacteriologic examination. This was achieved with no significant reduction in isolation rate or detectable increase in contamination.

The capability of isolating and classifying the fastidious microorganism Mycobacterium paratuberculosis has been developed through the

refinement of methods reported by other investigators. Recently a technique was introduced for culturing fecal specimens for M. paratuberculosis. This capability has provided the veterinarian with a means of obtaining culture information on living cattle.

Better methods have been developed for the inoculation of mediums for tuberculin production and several lots of tuberculin have been produced for experimental purposes. The responsibility of developing a production protocol for a standard lot of Purified Protein Derivative of M. bovis was assigned to the unit personnel.

Training has been an important function of the unit, which has contributed to the tuberculosis eradication program by conducting short courses for laboratory personnel and field veterinarians.

SAFETY MEASURES FOR USE IN THE MYCOBACTERIOLOGY LABORATORY

I. Health Program for Laboratory Workers

A. Pre-employment examination - tuberculin skin test (Mantoux) and Chest X-ray.

B. Employees working with tuberculosis should have a tuberculin skin test (Mantoux) every six months and a chest X-ray (14"x17") annually.

C. Employees may waive the tuberculin skin test if they are known to react positively but they should be required to have an annual chest X-ray at the discretion of the physician.

D. Employees associated with the laboratory, e.g., clerical workers, should be tuberculin skin tested (Mantoux) semi-annually and X-rayed annually.

II. Procedures in Contaminated Areas

A. Biological safety cabinets (hoods) with a filtered exhaust air system should be used for all activities involving viable mycobacteria. A negative pressure of 0.75 inch of water is maintained in the cabinet. Material to be removed from the hood is either decontaminated as a batch or individual articles are placed in an airlock at the end of the hood and decontaminated with a suitable disinfectant. A filtered air inlet is desirable to minimize contamination.

B. Pipettes are used only with a safety pipette bulb. (Materials are never pipetted by mouth.) All discarded pipettes are immersed in a suitable disinfectant. Contaminated pipettes should be sterilized by pressure steam sterilization 121° C for 30 minutes. Additional time must be allowed for large, dense volumes of material to be autoclaved.

C. Blenders should be used only in a safety cabinet. These devices can produce considerable aerosol and are very hazardous to use in a tuberculosis laboratory.

D. Culture tubes and petri dishes containing viable cultures should be opened only in a safety cabinet.

E. Animal rooms - Before entering animal rooms where animals are infected with mycobacteria, clothes should be changed (everything). Rubber gloves and a disposable hat should be worn. A surgical mask is worn over the mouth and nose. A plastic shield is worn over the eyes if animals are to be inoculated with live mycobacteria. Upon leaving the animal room, the individual should completely disrobe and take a complete shower, including hair. Clothes used in the animal room are autoclaved at 121° C for 45 minutes.

F. Syringes - Only syringes with a luer-lok fitting and a luer-lok needle are considered adequately safe.

III. Procedures for the Decontamination of Materials involved in Laboratory Accidents

In case of accidental exposure of pathogenic materials (e.g. infected specimens or cultures) the following precautions should be taken:

A. Do not breath for the few seconds required to don a simple face mask.* These masks are located in plastic bags at convenient places in each laboratory room.

B. Signal others to leave the room. Close doors.

C. Wait for external air supply mask.** The supply air must come from a compressor which does not utilize oil in the production of air pressure.

D. Cover spilled material and surrounding area with 4 percent Amphyl.***

E. Wait in the room for 15 minutes while disinfectant is acting. Maintain external air supply to face mask.

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* A simple face mask which excludes microorganisms but does not supply oxygen or external air is available from Mine Safety Appliances Company, Pittsburg, PA 15208.

** An external air supply mask is manufactured by Survivair, 3323 West Warner Avenue, Santa Ana, CA 92702.

*** Amphyl

Potassium Ricinoleate	44.0%
Ortho Phenylphenol	15.0%
Para-tert Amylphenol	6.3%
Alcohol	4.7%
Inert ingredients	30.0%

Manufactured by National Laboratories, Mountvale, NJ 07645.

F. Clean up disinfectant and debris.

G. Autoclave appropriate materials including contaminated clothing. Disinfect other materials by chemical disinfection.

H. Shower.

IV. Disinfectants

A. Disinfectants used in the laboratory are 4 percent Amphyl or 5 percent phenol. Amphyl is generally used to disinfect skin and phenol is used to disinfect equipment. Avoid the fumes of phenol.

B. Quaternary ammonium compounds should not be used in a tuberculosis laboratory as a tuberculocide. Benzalkonium chloride (Zephiran) is a notable example.

ISOLATION AND IDENTIFICATION OF MYCOBACTERIA: GENERAL

I. Processing Tissue Specimens

A. Accession number

An accession number is assigned to the specimens of each case upon arrival. The number accompanies the case during all laboratory processes and reporting.

B. Preliminary treatment of specimens

Tissue specimens are rinsed in a dilute solution of sodium hypochlorite* (1:1000) as soon as possible after receipt. All fat is removed from tissues using separate sterile scissors and forceps for each specimen. Avoid incising uncut lymph nodes.

The untreated tissues are submerged in fresh, 1:1000 sodium hypochlorite solution and allowed to stand undisturbed overnight at 4° C. The following day the tissues are washed a second time in fresh 1:1000 sodium hypochlorite solution.

C. Grinding and digesting (1,2)

The washed tissues are placed in sterile petri dishes and transferred to a biological safety cabinet where they are dissected with sterile scissors and forceps. The cut surfaces are examined for lesions and the observation recorded.

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* The dilute sodium hypochlorite solution is prepared by mixing 67 ml of 6.0% NaOCl with 3933 ml tap water.

The central area from each tissue specimen is transferred to a blender jar. Lesions are included with adjacent tissue. Fifty ml of nutrient broth containing 0.4% phenol red indicator is added to the blender jar. The mixture is exposed for two minutes to the action of the rotating blender knives. Inoculate four tubes of medium (as shown in Table 1) with untreated tissue suspension using cotton-tipped applicators. Transfer 5 ml of tissue suspension from blender jar to 13 x 100 mm tube and freeze at -20° C. Transfer 5 ml of tissue suspension from the blender jar to a 20 x 125 mm tube containing 5 ml of 0.5 N NaOH. Shake thoroughly (by hand). After standing for 10 minutes at room temperature, add 6 N HCl (one drop at a time using Pasteur pipette) until color changes to yellow. Neutralize using 1 N NaOH until pink color appears. Allow to stand for 5 minutes and check for color change of tissue suspension. Place tubes containing tissue suspension in air tight port and spray with 5% phenol. After standing for 15 minutes, remove tubes from port of biological safety cabinet and centrifuge at about 1650 RCF* for 30 minutes. Return tubes to biological safety cabinet and decant supernate (to about 1 ml). Inoculate 4 tubes of medium with sodium hydroxide treated sediment using cotton-tipped applicators (Table 1).

The sodium hydroxide treated sediment used to inoculate media should be kept for 24 hours at 4° C. This sediment may be used

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* RCF = Relative Centrifugal Force = $\frac{4 \cdot (3.14)^2 \cdot r \cdot n^2}{32.2}$

r = radius in feet.
n = revolutions per second.
Example: A centrifuge head which accommodates screw cap capsules is used at VSL. The extended capsules provide an effective radius of 9.25 inches. The RCF is 1641 when this equipment is operated at 2500 rpm.

to reinoculate media if tubes inoculated with untreated tissue suspension are contaminated.

If all inoculated slants are contaminated, it will be necessary to process the 5 ml aliquot of frozen tissue suspension using 2% NaOH for 20 minutes. Neutralize the tissue suspension and inoculate blood plates. Observe plates for bacterial growth at 24 hours and 48 hours. If no growth is observed, inoculate the 8 tubes of culture medium listed in Table 1.

D. Culture mediums

TABLE 1. Mediums Inoculated with Sodium Hydroxide Treated and Untreated Inoculums

Medium	Treated Inoculum	Untreated Inoculum
Stonebrink's	1	1
Middlebrook 7H-10 (with OADC & Sodium Pyruvate)	0	1
Herrold's (with glycerine) (with malachite green) (with mycobactin)	1	0
Herrold's (with glycerine) (with malachite green) (without mycobactin)	0	1
Herrold's (without glycerine) (without malachite green) (without mycobactin)	1	0
Lowenstein-Jensen (with glycerine)	0	1
Lowenstein-Jensen (without glycerine)	1	0

The tubes containing inoculated medium are sprayed with 5% phenol before removal from the safety cabinet. The tubes are placed in a rack so that a horizontal position (about 30 degrees elevation) is maintained during an overnight incubation period of 37° C. This step insures uniform contact of the inoculum over the entire surface of each slant. On the following day, the slants are returned to the vertical position for further incubation at 37° C.

E. Incubation and observation of slants

The incubating culture mediums are examined each week (for a period of 8 weeks) for the presence of mycobacterial colonies. If bacterial colonies resembling those of mycobacteria are found, a smear is made from each type. The smear is stained by the Ziehl-Neelsen technique and observed microscopically (oil immersion) for the presence of acid-fast bacilli. Each colony type of acid-fast microorganisms is used to inoculate a single tube of Dubos broth* containing Tween 80 and Dubos Oleic Albumin Complex. The broth is used at a later time for biochemical tests and to study cultural characteristics (see Figure 1).

II. Mycobacterial Identification Procedures

A. Observation of primary colonies

An understanding of the variations in colonial morphology resulting from different mediums or by subculturing is essential for the effective use of a mycobacterial typing scheme. Isolated colonies are observed for morphology and pigmentation with the aid of a hand lens or other optical device.

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* Difco Laboratories, Detroit, Michigan.

Phase	Mediums	Observations and Tests	Conclusions
I	Primary Colony	Primary colony is isolated.	A pure culture of acid-fast bacilli has been obtained.
	Dubos Broth	Typical mycobacterial growth develops in Dubos Broth. Microscopy of Ziehl-Neelsen smear reveals only red bacilli.	
II	P&B Fluid	1. Growth characteristics. 2. Cell length. 3. Niacin test. 4. Cording.	The information gained from these tests is usually sufficient to assign the following types: *M. bovis* *M. tuberculosis* *M. avium* complex* Runyon Group I* Runyon Group II* Runyon Group III* Runyon Group IV*
	Biochem	1. Growth in INH. 2. Growth in TCH. 3. Growth in Neotetrazolium. 4. Growth in SM 5. Growth in RIF. 6. Growth on 5% NaCl (17). 7. Tween hydrolysis.	
	7H-9 Fluid	1. Tellurite reduction.	
	L-J Slants	1. Appearance time. 2. Pigment production. 3. Colonial morphology. 4. Catalase activity. 5. Nitrate reduction** (17). 6. Arylsulfatase	
III	Dubos Broth 7H-10 Plates	The mycobacterial agglutination test is conducted.	The mycobacterial serotype of *M. avium* complex is determined.
IV	Dubos Broth	Chickens are inoculated and observed at necropsy for the presence (or absence) of pathologic changes.	Lesions are indicative of *M. avium* complex serotypes 1, 2 or 3. Absence of lesions is indicative of serotypes 4 through 20.

NOTE: Laboratory animal pathogenicity tests may be used to confirm other mycobacterial types (see Table 2).

* Mycobacterial agglutination test result is of value in final identification.

** Difco nitrate broth (Difco Laboratories, Detroit, Michigan).

Fig. 1. Mycobacterial typing scheme in four phases.

1. Mycobacterium bovis. Primary colonies of this species on Middlebrook's 7H-10 medium usually appear colorless, flat, irregular, rough and very dull by transmitted light. They appear flat and dry or resemble the convex, white, moist colonies of M. avium complex microorganisms and the unclassified mycobacteria on Herrold's medium. M. bovis isolates sub-cultured on Lowenstein-Jensen medium produce mammilate, colorless colonies (see Figure 2). M. bovis colonies growing on Stonebrink medium usually appear as white, moist, convex colonies resembling those of M. avium. Glycerine is generally detrimental to the primary growth of M. bovis: non-glycerinated mediums should be used.

2. Mycobacterium avium complex. Primary colonies of this complex usually appear moist, white and convex on all four types of mediums but transparent, opaque, rough and domed variants occur, especially on Middlebrook's 7H-10 medium.

3. Mycobacterium tuberculosis. Primary colonies are very dry, rough, raised, and white to buff with irregular edges when grown on Lowenstein-Jensen medium.

4. Runyon Group I, II, III and IV (4). Described in Table 2 and elsewhere in this manual. Certain serotypes of Runyon Group III bacteria previously referred to as M. intracellulare (3) are included in the M. avium complex.

Cellular and colonial characteristics of several mycobacteria and their respective biochemical reactions are presented in

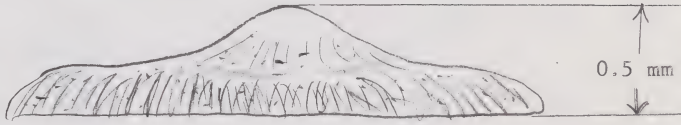


Fig. 2. Diagrammatic sketch of a typical M. bovis colony on Lowenstein-Jensen medium.

Table 2. A flow diagram illustrating the mycobacterial typing scheme is presented in Figure 3. A more detailed flow diagram of the same scheme is presented in Figure 1, depicting the four phases of the typing procedures.

B. Culture characteristics and biochemical tests

Smears are made of a representative of all colony types resembling those of mycobacteria. The smears are stained with the Ziehl-Neelsen procedure described in the appendix.

The remaining portion of each colony is transferred aseptically to a tube of Dubos broth (containing albumin and Tween 80). The broth is incubated for one week at 37° C. The purity of new growth is confirmed by smear and Ziehl-Neelsen staining. The incubation period is extended for another week if no growth appears.

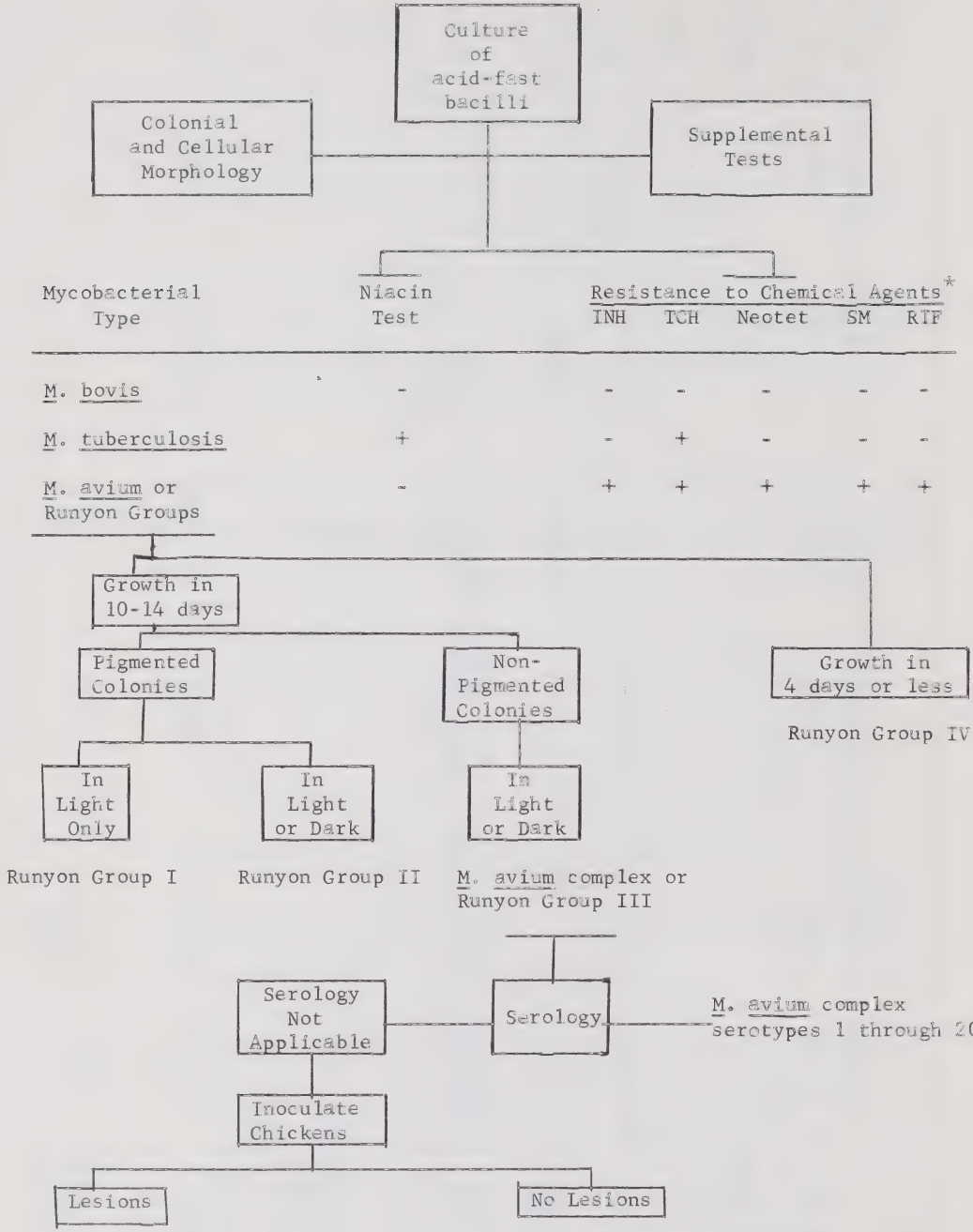
Both fluid and solid media are inoculated with the pure Dubos broth culture for use in subsequent typing procedures.

Proskauer and Beck medium (Karlson) is used to study four cultural characteristics in fluid medium.

1. Granular-uniform growth characteristics. Cells of M. tuberculosis and M. bovis cultures have a granular (clumped) appearance in transmitted light. All other mycobacteria usually have a uniform (homogenous dispersion of cells) appearance in transmitted light. Rough strains of M. avium and unclassified Runyon groups often produce clumps of cells in a background of uniform cells (see Figure 4).

TABLE 2.--Pertinent Characteristics of Three Mycobacterial Types and Four Unclassified Groups

		M. bovis	*M. avium* Complex	*M. tuberculosis*	Runyon Group I	Runyon Group II	Runyon Group III	Runyon Group IV
Appearance Time (Days)	at 37 C	13-20	13-20	13-20	13-20	13-20	13-20	6 or less
	at 45 C	No growth	6-13	No growth	No growth	No growth	±	Variable
Pigment Production	in light	-	-	-	+	+	-	Variable
	in dark	-	-	-	-	+	-	Variable
Cording		+	-	+	-	-	-	Variable
Growth Character in P&B Medium		Granular	Uniform to granular	Granular	Uniform	Uniform	Uniform to granular	Uniform to granular
Cell Length (microns) in P&B Medium		1.0 - 3.0	0.5 - 1.0	1.0 - 3.0	Variable	Variable	0.5 - 1.0	Variable
Colonial Morphology (on L-J slant)		Mammilate Irregular	Smooth, Convex Entire, Moist	Dry, Rough Heaped, Irregular	Smooth to rough Convex, Moist Entire	Smooth to rough, Convex Moist, Entire	Smooth, Convex Moist, Entire	Variable
Growth in INH		-	+	-	+	+	+	+
Growth in TCH		-	+	+	+	+	+	+
Growth in Neotetrazolium		-	+	-	+	+	+	+
Growth in Streptomycin		-	+	-	+	+	+	+
Growth in Rifampin		-	+	-	+	+	+	+
Niacin Test		-	-	+	-	-	-	-
Catalase Activity		Trace	Slight	Trace	Moderate	Moderate	Slight	Strong
Tellurite Reduction		-	+	-	-	-	+	+
Animal Pathogenicity	G. pigs	+++	-	+++	-	-	-	-
	Rabbits	+++	Yersin	-	-	-	-	-
	Chickens	-	+++	-	-	-	-	-
Number Serotypes		No test	20	No test	2	3		2



* See page 18 for description of chemical agents.

Fig. 3. Basic mycobacterial classification scheme.

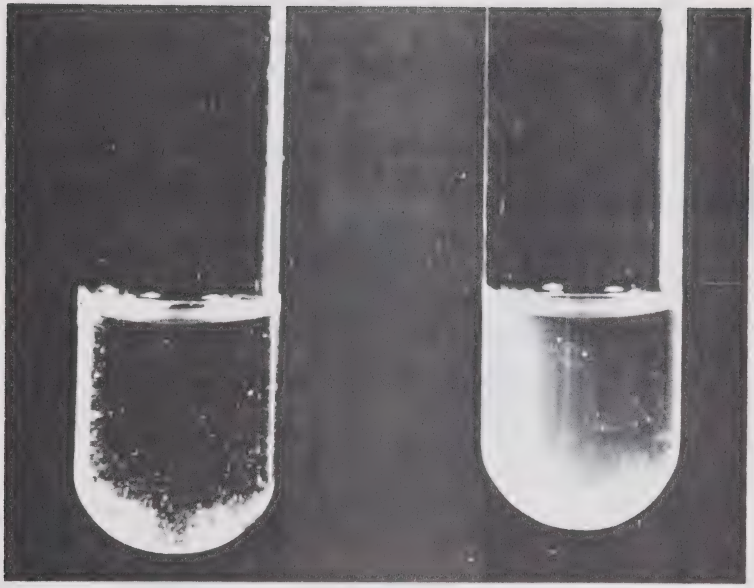


Fig. 4--Granular-uniform growth characteristics in Proskauer and Beck fluid medium. Granular growth with many clumped cells and clear background, left.

Uniform growth with occasional clumps and a homogenous dispersion of cells forming the background, right.

2. Cording. A smear of the bacterial sediment in a Proskauer and Beck culture is stained by Ziehl-Neelsen technique and examined by oil immersion microscopy for the presence of cords and for cellular morphology (see Figure 5).

Cording is a term used to describe a parallel alignment of bacterial cells that results in a long serpentine or string-like formation. M. tuberculosis and M. bovis produce cords consistently. The phenomenon is also reported to be an indicator of virulence, but avirulent cultures occasionally produce cording. Cords are most easily demonstrated in smears made from the condensate which collects at the butt end of the slant or in serum enriched fluid mediums.

3. Cellular morphology. This may be studied on the same smear and the observations used to aid in determining the bacterial type. The morphology of several mycobacteria is described in Table 2.

C. Biochemical tests

1. Niacin. The presence of niacin (5) in a culture growing in Proskauer and Beck medium may be used to differentiate M. tuberculosis from all other mycobacteria. M. tuberculosis is a vigorous producer of niacin. M. fortuitum has also been reported as a niacin producer.

One ml of 4.0% aniline in ethyl alcohol is added to the culture growing in Proskauer and Beck medium. This is followed

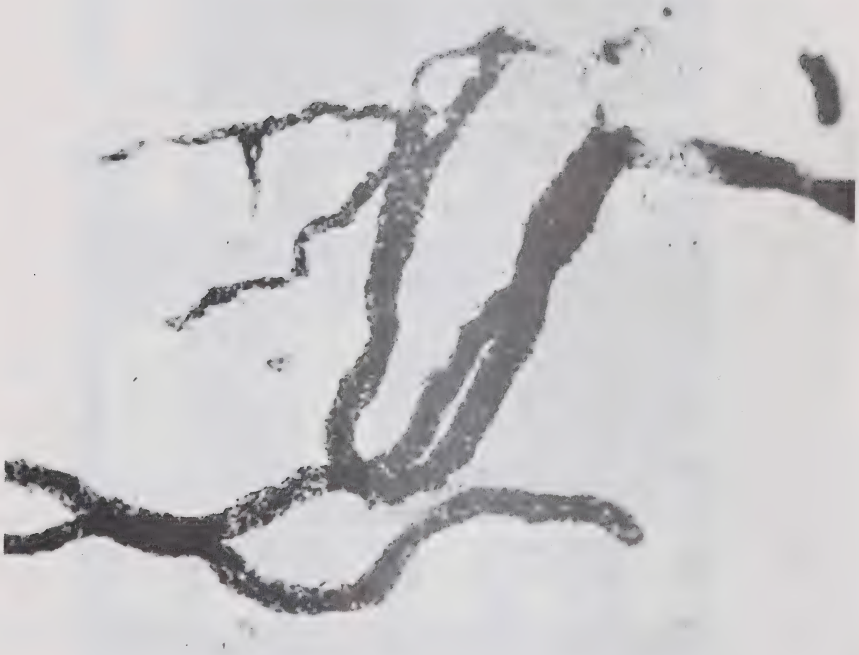


Fig. 5--Cording in a smear of M. bovis stained by the Ziehl-Neelsen method. Magnification 970X.

by the addition of 1.0 ml of aqueous cyanogen bromide (deadly poison) (see appendix). The appearance of a yellow color within five minutes is indicative of the presence of niacin.

2. Sensitivity (or resistance) to chemical compounds. (6) Sensitivity to a particular chemical compound is indicated when growth is inhibited in medium containing the agent but not in the control medium. Conversely, resistance to a chemical compound is elicited by bacterial growth in mediums containing the specific chemical. The sensitivity of mycobacterial cultures to iso-nicotinic acid hydrazid (INH) (17), thiophen-2-carboxylic acid hydrazid (TCH) (7), neotetrazolium chloride (neotet) (8), streptomycin (SM) (9) and Rifampin (RIF) (10) is essential in the classification scheme. Each chemical compound is incorporated into Dubos broth to obtain final concentrations of 10 μg/ml for INH and TCH, 1:40,000 for neotet, 2 μg/ml for SM and 0.250 μg/ml for RIF (see appendix). Each tube of medium and a control without chemical compound is inoculated with 0.1 ml of a heavy suspension of the culture to be typed. The sensitivity test is a fluid modification of the procedure used at the Center for Disease Control, Atlanta, Georgia.

Mycobacterial cultures which grow in the presence of neotetrazolium chloride produce formazans which impart a purple color to the culture.

M. bovis strains are inhibited by all five compounds but growth is luxurient in the control medium (see Figure 6).

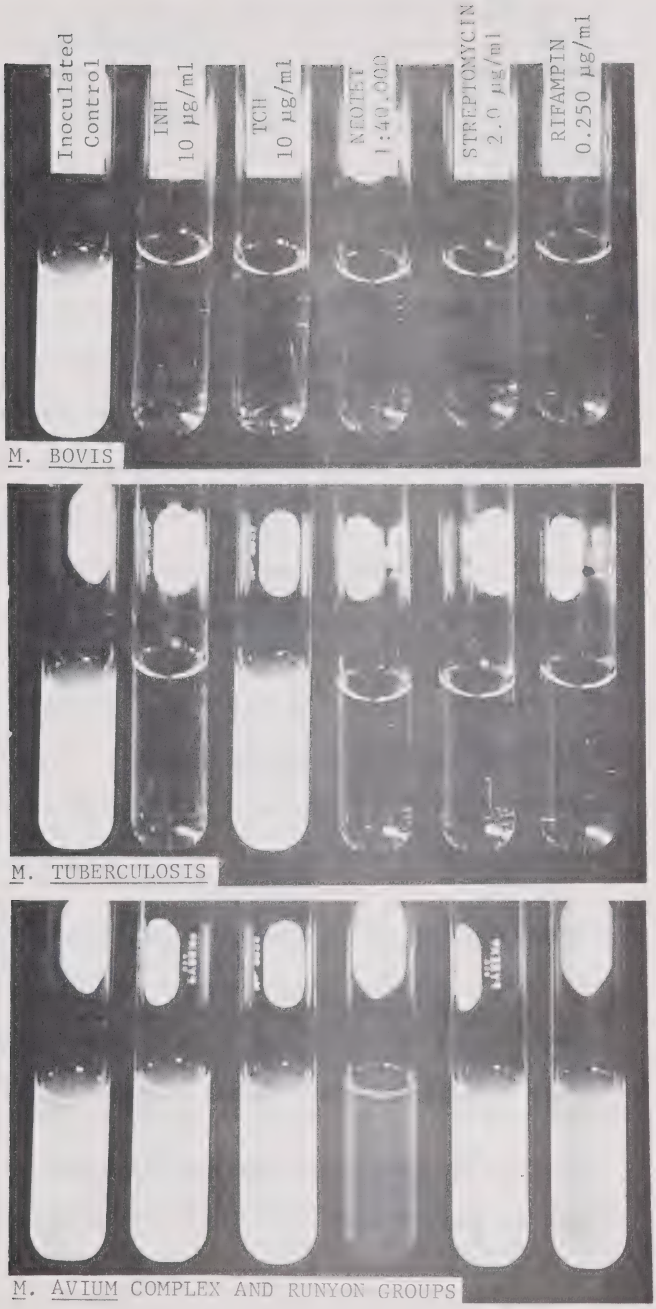


Fig. 6. Inhibition of mycobacteria by chemical compounds. Resistance is indicated by growth in appropriate tubes. Note pigmented growth of M. avium complex and Runyon groups in neotetrazolium.

M. tuberculosis strains are inhibited by INH, neotet, SM and RIF but grow well in TCH medium and control medium. *M. avium* complex and Runyon Groups I, II and IV are not usually inhibited by any of the five compounds. Therefore, luxurient growth occurs in mediums containing INH, TCH, neotet, SM, RIF and in the control medium.

D. Mycobacterial agglutination test (11)

1. Introduction. The mycobacterial agglutination test is useful in classifying certain cultures through the determination of their serotype. Mammalian tubercle bacilli do not produce stable suspensions and the test has not been applicable in their classification. Runyon Groups I, II and IV usually produce stable suspensions and a few serotypes have been described.

The greatest application of the agglutination test is found in differentiating the "*M. avium* complex." It can be seen in Figures 1 and 3 that cultural and biochemical techniques are inadequate for differentiating those organisms.

Antigen for the test is provided by a smooth, stable suspension of the bacterial cells to be serotyped. A standardized saline suspension of phenol killed cells is reacted with an equal volume of type specific antiserum, produced in rabbits.

A list of mycobacterial serotypes currently recognized is provided in Table 3.

Table 3. Mycobacterial Serotypes

Former Designations		Current Designations	
Mycobacterial Species	Serotype	Mycobacterial Species	Serotype
M. bovis	NA	*M. bovis*	NA
M. tuberculosis	NA	*M. tuberculosis*	NA
M. avium	1	*M. avium* complex	1
	2		2
	3		3
M. intracellulare	IV		4
	V		5
	VI		6
	VII		7
	Davis		8
	Watson		9
	IIIa		10
	IIIb		11
	Howell		12
	Chance		13
	Boone		14
	Dent		15
	Yandle		16
	Wilson		17
	Altman		18
	Darden		19
	Arnold		20
Runyon Group I	*M. kansasii*	*M. kansasii*	**
	M. marinum	*M. marinum*	
Runyon Group II	Scrofulaceum	*M. marianum*	41
(*M. scrofulaceum*)	Lunning		42
	Gause		43
Runyon Group IV	*M. fortuitum* I	*M. peregrinum*	**
	M. fortuitum II	*M. fortuitum*	**

* Wolinsky and Schaefer (12)

** Only one serotype recognized.

The procedure for conducting the mycobacterial agglutination test is presented below.

2. Preparation of cell suspension.

a. Make a needle transfer of the appropriate colony to a tube of Dubos broth containing DOAC and Tween 80.

b. Incubate the broth for 14 days at 37° C.

c. Transfer 1.0 ml of the fluid culture to each of two plates of Middlebrook's 7H-10 oleic albumin agar enriched with 4.1 gm of sodium pyruvate per 1000 ml of medium.

d. Incubate the inoculated agar plates for 14 days at 37° C

e. Check the agar surfaces and mycobacterial growth for evidence of contamination. If none is observed, add 2.0 ml of Phenolized Phosphate Buffered Saline (PPBS).* Use a rubber policeman to form a homogenous mixture on the agar surface.

f. Remove the homogenous mixture with a pasteur pipette.

g. Transfer a sufficient quantity of the cell suspension to produce a McFarland III cell density in 20 ml of PPBS

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* PPBS is composed of: Na_2HPO_4 0.14%
NaCl 0.8 %
Phenol 0.5 %
The pH is adjusted to 6.5 with 1N HCl. The pH increases to 7.0 during sterilization.

contained in a sterile 20 x 125 mm screw cap test tube. Allow the suspension to remain at room temperature for five days. (Phenol kills the cells during this period.) Rotate the tube occasionally to assure a smooth suspension of cells.

h. Sediment the suspension of killed cells by centrifuging at 2500 rpm for 30 minutes in a centrifuge with a 12-inch head.

i. Discard the supernate and add 2.0 ml of PPBS to the packed cells. Form a homogenous and concentrated suspension of cells by gently mixing.

j. Prepare a cell suspension of 0.4 Optical Density at 525 nm. The suspension is made by adding single drops of the harvested cells (in i. above) to PPBS in sterile 20 x 125 mm test tubes. The volumes of antigen and antiserum needed are dependent upon the number of tests to be made.

3. Test procedure.

a. Transfer 0.5 ml of the appropriate antiserum to an 11 x 100 mm glass test tube.

b. Add 0.5 ml of the properly diluted cell suspension to the antiserum and mix by gentle rotation.

c. Incubate the mixture at 37° C for 3 hours.

d. Observe the mixture for evidence of cell agglutination and clearing of the suspending fluid. Record observations using the numerical system described in Section 4.

e. Continue incubation for 15 additional hours.

f. Observe and record as in d. above.

4. Numerical designation of agglutination.

a. Three-hour reading:

4+ Large clumps of agglutinated cells throughout transparent suspending fluid.

3+ Medium sized clumps of agglutinated cells throughout slightly opaque suspending fluid.

2+ Small clumps of agglutinated cells throughout slightly opaque suspending fluid.

1+ Very small clumps of agglutinated cells throughout opaque suspending fluid.

0 No agglutinated cells. Suspending fluid is opaque.

b. Eighteen-hour reading:

4+ All cells are agglutinated and sedimented. Suspending fluid is transparent.

3+ Most cells are agglutinated and sedimented.

2+ Small clumps of agglutinated cells throughout slightly opaque suspending fluid.

1+ Very small clumps of agglutinated cells throughout opaque suspending fluid.

0 No agglutinated cells. Suspending fluid is opaque.

c. Rules for assigning mycobacterial serotype based on observations of agglutination:

Two antiserums representative of a single serotype must both cause agglutination of the unknown mycobacterial cells.

Agglutination must be observed at both the three and eighteen hour readings.

5. Selection of antiserums. The antigen used in this test is always a cell suspension of acid-fast bacilli of unknown mycobacterial type. The antiserum, however, varies as indicated by the selection listed in Table 3. The antiserums are produced in rabbits hyperimmunized with aliquots of phenol killed cells of known mycobacterial type prepared as described under Section 2., "Preparation of cell suspension."

The detailed procedures for determining potency and cross agglutination characteristics and serum adsorption techniques may be obtained on request from this laboratory.

a. Screening serotypes:

The alert serologist will determine from test data the serotypes which are more likely to occur in the particular mycobacterial flora being isolated. Considerable effort can be conserved by establishing a battery of

screening serotypes composed of those most frequently isolated.

The following sequence of mycobacterial antiserums is used at this laboratory for testing isolates which grow well at 37° C, usually grow at 45° C, appear on primary isolation at about 13 days, are resistant to INH, TCH, neotet, SM and RIF, show no cording, weak catalase, and whose cells are short, coccoidal acid-fast rods:

Two antiserums representing M. avium complex serotype 1.

Two antiserums representing M. avium complex serotype 2.

Two antiserums representing M. avium complex serotype 4.

Two antiserums representing M. avium complex serotype 8.

b. Remainder of recognized serotypes (use two antiserums representing each):

M. avium complex serotypes 3, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.

c. Other serotypes:

The following mycobacterial antiserums are used if preliminary typing results indicate Runyon Group I microorganisms (use two antiserums representing each serotype)

M. kansasii.

M. marinum.

The following mycobacterial antiserums are used if preliminary typing results indicate Runyon Group II microorganisms (M. marianum) (use two antiserums representing each serotype):

Serotype 41, 42 and 43

The following mycobacterial antiserums are used if preliminary typing results indicate Runyon Group IV microorganisms (use two antiserums representing each serotype):

M. peregrinum

M. fortuitum

6. Alternative procedures. Suitable suspensions of some mycobacterial isolates are not obtainable with present techniques. Their identification may be made by using animal pathogenicity tests and supplemental in vitro tests (13).

E. Supplemental tests

1. Catalase. The catalase test (14) provides a measure of the enzyme "catalase" produced by mycobacterial isolates. It can be a useful adjunct in the classification scheme. Clinically significant strains are generally light producers of the enzyme. Clinically insignificant strains generally produce the enzyme in much greater amounts.

M. bovis, M. avium complex and M. tuberculosis are notable examples of light producers.

The test procedure is described below:

Add 2.0 ml of catalase reagent* to a culture on Lowenstein-Jensen medium with glycerine incubated in a 20 x 125 mm test tube at 37° C for four weeks.

Observe after five minutes for the release of gas as indicated by small bubbles formed at the surface of the colonies and on the fluid normally occurring at the base of the slant.

A column of bubbles is measured and the results described by the following terms:

Weak	Column less than 10 mm
Strong	Column 10 to 50 mm
Very Strong	Column greater than 50 mm

2. Tellurite. The tellurite test (15) is a measure of the ability of mycobacteria to reduce colorless potassium tellurite to black, metallic tellurium. The test may be used as an adjunct in the differentiation of Runyon Group IV strains from pathogenic mammalian strains (see Table 2). The test procedure is described below:

Inoculate a tube of Middlebrook's 7H-9 medium with the suspect culture.

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* Catalase reagent consists of a 1:1 mixture of 10% Tween 80 and 30% Hydrogen Peroxide.

Incubate for 1 week at 37° C.

Add 2 drops of sterile aqueous 0.2% potassium tellurite solution to the culture.

Return the culture to the incubator and examine in 3 days for reduction of the tellurite salt to the black, metallic tellurium.

The majority of M. avium complex and most Runyon Group IV mycobacteria reduce tellurite in 3-4 days. Other mycobacteria fail to reduce tellurite during this period.

3. Arylsulfatase test. The arylsulfatase test (16,17) is useful in differentiating M. fortuitum and M. chelonei from slowly growing mycobacteria. The test is useful in separating M. xenopi from other nonphotochromogenic mycobacteria. M. fortuitum and M. chelonei give a positive reaction while other mycobacteria are usually negative. M. xenopi is variable but generally positive.

The substrate is prepared by mixing 2.5 ml of filter sterilized 0.08 M potassium phenolphthalein disulfate (stock solution) to 200 ml of Dubos broth with albumin: providing a 0.001 M working solution. The sterile working solution is transferred, in 2 ml aliquots, to 16 x 125 mm screw cap test tubes.

The test is conducted by transferring a loopful of 1-2 week old culture (from a slant) to a test tube containing 2 ml of working solution. The mixture is incubated for 3 days at 37° C. Six drops of 2 N sodium carbonate is added causing the development of a red color in the substrate (positive) or no color change (negative). The color intensity is recorded using an arbitrary scale of 1+ (weak) through 5+ (strong).

4. Tween 80 hydrolysis (17,18). This test aids in the differentiation of clinically significant and saprophytic isolates of M. avium complex and Runyon groups II and III.

The substrate medium is prepared by combining 100.00 ml of M/15 phosphate buffer* at pH 7, 0.5 ml of Tween 80 (polyoxyethylene sorbitan monoleate), 2.0 ml of a 0.1% aqueous neutral red solution. The mixture is dispensed in 2.0 ml aliquots into 16 x 125 mm screw cap test tubes and autoclaved for 10 minutes at 121° C. The substrate may be stored at 4° C for 2 weeks without loss of reactivity.

Isolates are tested by transferring a large loopful (3 mm) of growth from a slant to 2.0 ml of tween substrate. Observations are made at 10 days of incubation at 37° C. Strains which hydrolyze Tween 80 also cause an alkaline condition in the substrate which develops a pink to red color in the normally amber substrate.

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* Phosphate Buffer: Na_2HPO_4 9.47 gm in 1000 ml H_2O - 61.1 ml
KH_2PO_4 9.07 gm in 1000 ml H_2O - 38.9 ml

F. Miscellaneous evaluations of isolates

M. bovis and M. tuberculosis cultures are differentiated on the basis of growth characteristics in Proskauer and Beck medium, niacin test, sensitivity to chemical agents and cellular morphology.

Colonies of M. avium complex and Runyon Group III strains appear in 13 to 20 days at 37° C. M. avium complex serotypes 1, 2 and 3 appear in 7 to 14 days at 45° C, whereas the appearance time of the other serotypes is variable. Pigmentation is usually absent. The colonies are moist, white, entire and convex. M. avium complex cultures can usually be differentiated by the mycobacterial agglutination test. If a culture is not suitable for use in the serologic test, final differentiation may be made by animal pathogenicity tests. M. avium complex serotypes 1 and 2 usually cause generalized lesions in the liver, spleen and kidneys of chickens.

Both Runyon Groups I and II produce orange to yellow pigment under certain light conditions. Group I colonies are pigmented when grown in light but colorless when grown in darkness. Group II colonies are pigmented when grown in light or in darkness.

The light requirement for the differentiation of Group I and Group II colonies can be satisfied if the culture tubes occupy a position near an incubator door which is opened several times a day. Satisfactory conditions of darkness can be produced by covering the appropriate culture tubes with aluminum foil.

Runyon Group IV microorganisms are characterized by bacterial growth appearing in six days or less at 37° C. Temperature

requirements for growth are not restrictive with growth occurring from room temperature to 45° C for many strains. Pigment is not usually present in colony growth but may occur occasionally.

G. Animal pathogenicity tests

The inoculation of laboratory animals may be used as an adjunct to the cultural, biochemical and serologic typing techniques presented in this manual. M. tuberculosis, M. bovis, M. avium complex and Runyon Group III isolates may be differentiated by this method. Other mycobacteria are not typed by this method because they are not normally virulent for laboratory animals.

The virulence of several mycobacteria for selected laboratory animals is presented in Table 2.

Guinea pigs and chickens are each inoculated intraperitoneally with 1.0 ml of a suspension containing 0.1 mg of bacterial cells per ml of Butterfield's buffer. Rabbits are inoculated intraperitoneally with 1.0 ml of suspension containing 0.01 mg of bacterial cells per ml of Butterfield's buffer.

Inoculated animals are kept for two months. Any that die within that period of time are necropsied and observed for gross evidence of tuberculosis. All surviving animals are necropsied at the end of the two-month period and similarly observed.

The concentration of bacterial suspensions for animal inoculation may be determined spectrophotometrically by determining the optical

density (at 525 nm) of cell suspensions and making appropriate dilutions to the prescribed concentration. A reference curve correlating cell concentration with optical density must be prepared for each mycobacterial species prior to standardization of unknown cultures.

If a spectrophotometer is not available, a Fitch-Hopkins calibrated centrifuge tube may be used to estimate the concentration (wet weight) of mycobacterial cells in suspension (19).

ISOLATION AND IDENTIFICATION OF M. PARATUBERCULOSIS

Mycobacterium paratuberculosis is the etiologic agent of Johne's Disease which is most prevalent in cattle, sheep and goats. Other ruminants and swine may be infected with M. paratuberculosis but it is innocuous for man. The infection involves the intestinal canal and lymph nodes of the mesentery. The terminal ileum, ileocecal valve and adjacent cecum are most frequently infected.

M. paratuberculosis organisms are vastly outnumbered by other bacteria in fecal and intestinal tissue specimens. The primary cultivation of M. paratuberculosis was considered impractical before Merkal, et al. (20) developed effective culture methods. The organism is a small, gram positive, acid-fast rod. It usually occurs in clumps in both tissues and feces. Primary colonies are very small, translucent, and glistening with entire margins.

I. Processing Tissue Specimens

A. Identification of specimens

An accession number is assigned to the specimens of each case upon arrival. The number accompanies the case during all laboratory processes and reporting.

B. Preliminary treatment of tissues

Most tissue specimens from which M. paratuberculosis is isolated are portions of the intestinal canal. The feces should be rinsed from them before shipment to the laboratory but this should be repeated at the laboratory to avoid unnecessary contamination. The tissues should be shipped frozen. Chemical preservatives

should not be used. The tissues are thawed the day before processing, rinsed in Butterfield's Buffer, and held overnight in fresh Butterfield's Buffer.

C. Selection and digestion of tissues

The tissues are rinsed again in fresh Butterfield's Buffer the next day. Approximately four grams of mucosa are scraped from the ileocecal valve or other areas showing pathologic change (e.g., thickening, erosions, inflammation) and placed in a sterile blender jar containing 50 ml of 2½% Trypsin. The mixture is adjusted to neutrality using 4.0% sodium hydroxide and pHydrion paper and stirred for 30 minutes at room temperature on a magnetic mixer. The digested mixture is filtered through gauze supported by a small funnel

The filtrate is centrifuged at approximately 1650 RCF for 30 minutes. The supernatant fluid is poured off and discarded.

D. Decontamination of inoculum

The sediment is resuspended in 40 ml of 0.1% Zephiran and allowed to stand undisturbed for 24 hours. The particles which settle to the bottom of the tube are referred to as the inoculum which is removed by pipette without disturbing the supernatant fluid.

E. Inoculation of culture mediums

Sterile pasteur pipettes are used to transfer approximately 0.1 ml of inoculum to each of three slants of Herrold's medium containing

mycobactin and to one slant of Herrold's medium without mycobactin (Some microbiologists prefer to inoculate equal numbers of slants with and without mycobactin because the occurrence of growth on either medium is a significant factor in identifying the isolate.) The inoculum is distributed evenly over the surface of the slants. The tubes are allowed to remain in a slanted position at 37° C for approximately one week with screw caps loose.

The remaining inoculum is retained at room temperature in the original test tube containing approximately 40 ml of 0.1% Zephiran. This reserve inoculum may be used to inoculate fresh medium in case of excessive contamination of inoculated slants. The need for the reserve inoculum is usually known within one week after the initial inoculations.

The tubes are returned to a vertical position when the free moisture has evaporated from the slants. The lids are tightened and the tubes are placed in baskets in an incubator operated at 37° C.

F. Incubation and observation of slants

The slants are observed one week after inoculation. If fungal or bacterial contaminants have overgrown any of the four slants of a single case, the reserve inoculum is used to inoculate fresh Herrold's medium to replace the discarded slants.

The inoculum is used without additional treatment. The non-acid-fast bacterial population is reduced during storage by the selective

bacteriocidal activity of Zephiran. If contamination re-occurs, the original Zephiran is removed and 40 ml of 0.3% Zephiran is added to the inoculum. The Zephiran and inoculum are mixed and allowed to stand undisturbed for 24 hours. The re-treated sediment is used to inoculate fresh medium. Mycobacteria may be lysed by Zephiran if the contact time is prolonged. The bacteriostatic effect of Zephiran is inactivated by phospholipids (21,22,24). The egg in Herrold's medium contributes sufficient phospholipids to neutralize the bacteriocidal activity of residual Zephiran in the inoculum. Agar mediums (e.g., Middlebrook's 7H-10) do not provide this protection.

Zephiran is relatively ineffective in controlling the growth of contaminating fungi. Conversely, amphotericin B (Fungizone*) was found by Merkal et al. (23) to effectively control fungal overgrowth of inoculated media.

Fungizone may be incorporated in the Herrold's medium at a final concentration of 50 µg per ml of media. Due to loss of antifungal activity, storage of Herrold's medium containing Fungizone should be limited to one month at 4° C. Storage at ambient temperatures is not recommended.

Fecal specimens from cattle which are known to contribute fungal contamination may be treated with Fungizone before the medium is inoculated. The inoculum (sediment described in D. above) is

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* E. R. Squibb and Sons, Inc., New York, New York.

treated at the rate of two drops of 5.0 mg per ml Fungizone added to each ml of sediment, mixed and transferred, with the sediment, to medium slants.

The slants are observed bi-weekly for 15 weeks. Contaminated slants are discarded and all colonies resembling those of mycobacteria are selected for identification. All uncontaminated slants are incubated for the entire period even though mycobacterial colonies may have been isolated earlier.

G. Identification tests

1. Appearance time. Primary colonies of M. paratuberculosis may be expected to appear any time from the fifth to the fourteenth week after inoculation. Most colonies appear during the seventh week, but many appear during the fifth and sixth weeks.

2. Colonial morphology. Primary colonies on Herrold's Egg Yolk Agar Medium with mycobactin are very small (1 mm diameter), colorless, translucent and hemispherical. Their margins are round and even. Their surfaces are smooth and glistening. The colonies become more opaque and increase in size as incubation is continued. Pigmentation is never observed in primary colonies. Isolated colonies may increase in diameter to 4 or 5 mm. Roughness increases with age and, like many mycobacteria, M. paratuberculosis becomes rough when subcultured on Middlebrook's 7H-10 medium with mycobactin. The colonial morphology changes with age from hemispherical to mammilate. The round and even margin is maintained regardless of incubation time.

3. The cells of primary colonies of M. paratuberculosis are highly acid-fast and Gram positive. The cells average 0.5 μ in diameter and 1.0 μ in length. Spores and capsules are absent. They develop intracellularly in epithelioid and giant cells at the site of localization in tissue. They generally occur in clumps in both tissue and feces.

4. Mycobactin dependency (25,26,27). Primary colonies of M. paratuberculosis exhibit a strict dependency upon mycobactin (an extract of M. phlei) for growth. Subcultures occasionally lose their mycobactin dependency with successive transfers. Slants of Herrold's medium without mycobactin will support subdued growth of M. paratuberculosis in rare instances. This modification of mycobactin dependency is more prevalent in subcultures than in primary cultures. The growth is always less luxurient than that occurring on the Herrold's medium with mycobactin similarly inoculated. Great numbers of M. paratuberculosis in the inoculum are believed to provide enough mycobactin to support the minimal growth on Herrold's medium without mycobactin. If there is distinct evidence of mycobactin dependency shown by the primary isolate and if all other tests are compatible, it may be reported as M. paratuberculosis. If mycobactin dependency is absent or indistinct, a single typical colony is subcultured on two slants of Herrold's medium containing mycobactin and two slants of Herrold's medium without mycobactin. Several examples of mycobactin dependency

which may be used as guidelines for subculturing are shown in Table 4.

H. Supplemental tests

The biochemical and supplemental tests commonly used to identify other mycobacteria have been investigated (28) and found to be inconclusive for use with M. paratuberculosis.

I. Animal pathogenicity tests

M. paratuberculosis organisms do not cause progressive disease in guinea pigs, hamsters, rabbits, mice or chickens. The results of such animal inoculations would be of little value in diagnostic laboratory activities.

Ruminants are susceptible to M. paratuberculosis. Sheep, for example, may be drenched with cream containing 200 mg dry weight of viable M. paratuberculosis (29). Clinical symptoms may appear in eight to fourteen months. The cost of ruminants, the expense of housing them and the chronic nature of the disease make them impractical for diagnostic purposes or identification of the organism.

J. Miscellaneous aspects

1. Discoloration of Herrold's medium. Herrold's medium is normally light green in color. That color is usually maintained after inoculation but in some instances the slant becomes yellow when colonies of M. paratuberculosis appear. In other instances, the color may change from green to yellow in the absence of bacterial growth. The color change also

TABLE 4.--Examples of Culture Results and Corresponding Recommendations for Subculturing and Reporting.

Examples of Culture Results	Mediums* M	M	M	C	Subculture	Final Report
Typical colonies greater than 10 in number, on all mycobactin enriched slants. No typical colonies on control slant.	+	+	+	-	No	- at end of primary isolation period. Report sent out immediately.
Typical colonies on all mycobactin enriched slants. No typical colonies on control slant.	7	6	7	-	Yes	When results of subculturing are available.
Typical colonies on all mycobactin enriched slants. No typical colonies on control slant.	1	1	2	-	Yes	When results of subculturing are available.
Typical colonies on two mycobactin enriched slants. No typical colonies on control slant.	1	2	-	-	Yes	When results of subculturing are available.
Typical colonies on two mycobactin enriched slants. No typical colonies on control slant.	+	10	-	-	Yes	When results of subculturing are available.
More than 10 colonies on each of the 4 slants.	+	+	+	+	Yes. Also perform identification tests for other mycobacteria.	When results of subculturing and identification tests for other mycobacteria are available.

* M = mycobactin in the medium.
C = control slant without mycobactin
+ = more than 10 colonies visible on the slant.
Numerals = indicate the number of colonies appearing on a slant.

occurs in uninoculated slants stored at room temperature. A color change in the medium without the appearance of colonies should never be considered indicative of M. paratuberculosis.

Contaminating fungi usually overgrow the surface, imparting the color of hyphae or spores characteristic of the fungal species.

Bacterial contaminants generally impart a gray-blue color and frequently liquefy the medium.

Excessive acidity in the inoculum may impart a dense blue-green color to the medium. This condition has been noted frequently when the inoculum is derived from goat feces. It may be corrected by adding eight to ten drops of phosphate buffer* to the inoculum after it has been treated with Zephiran.

2. Pitting. Fresh Herrold's medium may or may not have pits in the surface of the slant. The cause of this condition is not fully understood but it is believed to be due to the presence of minute air bubbles in the molten medium at the time the slant is formed. When the bubble bursts, a minute pit remains. These pits can be mistaken for very early colonies of M. paratuberculosis. It is believed (but unproven) that many colonies of M. paratuberculosis evolve from pits in the inoculated medium.

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* Phosphate Buffer: Na_2HPO_4 14.2 gm
NaCl 80.0 gm
Distilled water QS to 1 liter

3. Other isolates. Some non-acid-fast bacteria survive the decontaminating action of Zephiran and produce colonies on the Herrold's medium with or without mycobactin. These isolates are easily rejected by observing smears stained by the Ziehl-Neelsen method.

Other mycobacteria may be isolated and cultured by the methods described in this manual. Ziehl-Neelsen staining indicates their acid-fast character but does not aid in determining the mycobacterial type. Mycobactin dependency is most useful in separating M. paratuberculosis from other mycobacteria which are culturable on Herrold's Egg Yolk Agar medium.

II. Processing Fecal Specimens

A. Collection, shipment and identification

The fecal specimen is taken from the rectum using a dry single service glove. Approximately one half ounce of feces is placed in a one-ounce ointment tin. The tin is identified, sealed with masking tape and shipped in an insulated container to the laboratory in the fastest way. No refrigerant or chemical preservative is used. A submission form listing each animal number and tin should accompany the shipment.

An accession number is assigned to the group of fecal specimens from one herd. Individual case numbers may be assigned to each fecal specimen to facilitate identification in the laboratory. These identification numbers accompany the case during all laboratory processes and reporting.

B. Suspension and decontamination of feces

Approximately one gram of feces is transferred with a sterile wooden tongue depressor to a 50 ml centrifuge tube containing 40 ml of sterile distilled water. The mixture is shaken for 30 minutes at room temperature. The larger particles are allowed to settle for 30 minutes. The uppermost 5.0 ml of feces suspension is transferred to a 50 ml centrifuge tube containing 35 ml of 0.3% Zephiran. The tube is inverted several times to assure uniform distribution and allowed to stand undisturbed for 24 hours at room temperature.

C. Inoculation of culture mediums

A sterile Pasteur pipette is used to transfer 0.1 ml of the undisturbed sediment to each of four slants of Herrold's Egg Yolk Agar (three with mycobactin and one without mycobactin).

D. Preparation of smears

A smear may be made from the sediment and stained by the Ziehl-Neelsen method. The presence of acid-fast bacilli in smears of feces is not diagnostic for Johne's disease because saprophytic acid-fast bacilli are normally present in feces of infected and healthy cattle.

M. paratuberculosis organisms frequently occur as clumps in the feces of infected animals but this characteristic is unreliable and should not be used as a major factor in the laboratory identification of the organism.

E. Incubation and observation of slants

Same as for tissue (see page 36).

F. Identification tests

Same as for tissue (see page 38).

G. Supplemental tests

Same as for tissue (see page 40).

H. Animal pathogenicity tests

Same as for tissue (see page 40).

I. Miscellaneous laboratory aspects

Same as for tissue (see page 40).

ACKNOWLEDGEMENT

The authors thank Drs. A. B. Larsen and R. S. Merkal, Tuberculosis Unit, Bacteriological and Mycological Investigations, Veterinary Research Services, ARS, USDA, for their invaluable assistance in writing the procedures for the Isolation and Identification of M. paratuberculosis.

APPENDIX

LOWENSTEIN-JENSEN MEDIUM*

Ingredients	For 1612 mls Media
Lowenstein Medium Base (Difco 0444)	37.2 gm
Glycerol	12.0 ml
Distilled Water	600.0 ml
**Whole Eggs - aseptically prepared	1000.0 ml (24 large eggs)

Equipment Needed

Prepare and sterilize:

One 2 liter aspirator bottle with small bell filling attachment for dispensing and preferably a magnetic stirring bar for mixing.

Large funnel - lined with two thicknesses of unbleached muslin. Tape edge to hold in place. Huck towel folded over muslin lined funnel. Tape in place.

Two quart Ball jars with Osterizer blender tops. Autoclave in inverted position.

Funnel wrapped in paper for sterilizing.

Test tubes - 20 x 125 screw cap tubes in sufficient quantity.

Pipette or glass rod for stirring eggs.

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* This media is also made without Glycerol when specified.

** Eggs should be strictly fresh. Eggs must be from hens that have had no antibiotics in feed or medicine.

Method of Preparation

Add Lowenstein medium base to water containing glycerol. Mix on Waring blender to obtain a smooth solution. Heat in flask until dissolved. Pour into a 2 L. aspirator bottle. Sterilize in an autoclave for 20 minutes at 121° C. Cool to room temperature.

Egg Preparation

Scrub eggs in warm detergent water, rinse in clear water and air dry on towel. Soak in 75% isopropyl alcohol for 30 minutes. Dry eggs between layers of sterile towels. Dip top of beaker in alcohol and flame dry to sterilize. Break eggs on sterile beaker edge and drop in sterile Ball blender jars. Mix on Osterizer just enough to homogenize but not to cause excessive air bubbles. Filter through sterile muslin lined funnel into sterile media in aspirator bottle. (Put gauze plug from bottle inside towel layer to keep sterile while eggs are filtering.) Stir eggs with sterile pipette or rod if necessary to help filtering process but keep eggs in funnel covered with towel gauze plug in aspirator bottle. Mix gently but thoroughly on magnetic mixer until media is evenly blended. Use aseptic technique in dispensing medium. Medium should flow into the tube on the etched side. Do not shake tube after filling to avoid film on more than bell side. Dispense in 9 ml amounts in 20 x 125 screw cap tubes. Dispense on bell side of tube only. Inspissate for 40 minutes at 80° C with tubes in slant position. Cool slightly. Incubate 24 to 48 hours in slant position so excess moisture will be reabsorbed by media.

STONEBRINK'S MEDIUM

Ingredients	For 1200 ml Total Media
Salt Mixture	
Sodium Pyruvate (Sodium Salt-Pyruvic Acid)	5.0 gm
Potassium Phosphate Monobasic KH_2PO_4	2.0 gm
Distilled Water	300.0 ml
Sodium Phosphate Dibasic (Na_2HPO_4) to obtain pH of 6.5	
Dye Mixture	
Crystal Violet	100.0 mg
Malachite Green (oxalate form)	800.0 mg
Distilled Water	100.0 ml
Egg Mixture	
*Whole Egg (aseptically prepared)	800.0 ml (20 eggs)

This makes an average of 140 slants of media.

Equipment to Prepare and Sterilize

1 - 250 ml screw cap flask.

1 - 2 Liter aspirator bottle with magnetic stirring bar within a small bell filling attachment on rubber hose to dispense aseptically into tubes.

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* Eggs should be strictly fresh. Eggs must be from hens that have received no antibiotics in feed or medication.

Equipment to Prepare and Sterilize continued . . .

1 - Large funnel lined with 2 layers of unbleached muslin and covered with huck towel (muslin taped in place), then wrapped in paper and taped to sterilize.

2 - Quart jars with Osterizer blender tops, sterilized in inverted position.

1 - Pipette or glass rod to stir eggs in funnel

Tubes - 20 x 125 screw cap in sufficient quantity.

Method of Preparation

Mix salt solution until completely dissolved. Adjust pH to 6.5 with Sodium Phosphate Dibasic. Place in aspirator bottle. Mix dye solution until completely dissolved. Place in screw cap flask. Sterilize by autoclaving at 121^{o} for 20 minutes. Cool to room temperature.

Prepare Eggs as Follows:

Wash with brush in warm detergent water, rinse in clear water and lay out on towel to dry. Soak in 75% isopropyl alcohol solution for 30 minutes. Dry by inserting between layers of sterile towels. Dip top of a beaker in alcohol. Burn off alcohol with bunsen burner to sterilize. Break eggs on sterile edge of beaker, drop into sterile blender jars. Mix gently on Osterizer just enough to homogenize egg mixture but not enough to cause air bubbles. Add dye mixture to salt mixture in the aspirator bottle. Filter eggs through sterile muslin lined funnel into solutions in the aspirator bottle. Put cotton gauze plug from bottle between layers of the huck towel while eggs are filtering through. Stir eggs with sterile pipette or rod if necessary to help filtering process and keep eggs in funnel covered with

Preparation of Eggs continued . . .

towel as much as possible. When egg mixture has completed filtering, then replace cotton gauze plug in aspirator bottle and mix gently but thoroughly on magnetic stirrer, until the media is evenly blended. Dispense into tubes aseptically. Dispense on bell side of tube only and do not shake tube after filling to avoid film on more than bell side. Dispense in 9 ml amounts in 20 x 125 screw cap tubes. Inspissate for 40 minutes at 80° C with tubes in slant position. Cool slightly. Incubate 24 to 48 hours in slant position so excess moisture will be reabsorbed by media.

HEN EGGS FOR HERROLD'S EGG YOLK AGAR MEDIUM

The eggs used in the production of Herrold's Egg Yolk Agar Medium contain no antibiotics. To insure this, antibiotics must be withheld from the hen's ration. It is generally difficult to find a source of eggs guaranteed to contain no antibiotics. Two sources of suitable eggs, as of January 1, 1974, are given below:

SPAFAS, Inc.
Roanoke, Illinois 61561

SPAFAS, Inc.
Route 3
Norwich, Connecticut 06360

The price of specific pathogen free eggs is $3.00 per dozen in case lots, F.O.B. Roanoke or Norwich.

HERROLD'S EGG YOLK MEDIUM* (30)

Ingredients	For 1020 mls Media
Peptone (Difco 0118)	9.0 gm
Sodium chloride	4.5 gm
Agar (Special Noble-Difco 0.142)	15.3 gm
Beef Extract (Difco 0126)	2.7 gm
Glycerol	27.0 ml
Distilled Water	870.0 ml
Egg Yolks	6
Aseptically prepared from strictly fresh eggs. Eggs must be from hens that have had no antibiotics in feed or medicine.	
2% Malachite Green Dye Solution (aqueous) (oxalate form)	5.0 ml

Equipment

Prepare and sterilize:

One 2 Liter aspirator bottle with cotton gauze plug; small bell filling attachment for dispensing and preferably a magnetic stirring bar for mixing. Rat tooth forceps for each egg.

Test tubes - 20 x 125 screw cap tubes in sufficient quantity.

Method Preparation

Mix and heat all ingredients (except egg yolks and dye solution) until agar is melted. Cool to 60° C and adjust pH to 7.5 with 1 N. Sodium

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* This medium is also prepared without Glycerol and Malachite Green.

Method of Preparation continued . . .

Hydroxide. Pour into aspirator bottle and sterilize in autoclave 25 minutes at 121° C. Cool to 56° C and add six sterile egg yolk prepared as follows:

Scrub eggs with a brush in warm detergent water and rinse with clear water. Let air dry on a towel. Soak in 75% isopropyl alcohol for 30 minutes. Dry between layers of sterile towels. Crack shell at one end with sterile forceps, make 10 mm hole and remove egg white with forceps and gravity. Make hole larger and break yolk. Twirl forceps to mix yolk, pick out yolk sac with forceps and pour mixed yolk into sterile media in aspirator bottle. Repeat process for each egg. Mix media gently on magnetic mixer. Add Malachite Green dye solution with sterile pipette. Blend again on mixer. Dispense aseptically into 20 x 125 screw cap tubes in 9 ml amounts. Dispense on bell side of tube only. Do not shake tube after filling to avoid film on more than bell side. Allow medium to harden in slant position. Check sterility by incubation for 48 hours at 37° C.

HERROLD'S EGG YOLK MEDIUM WITH MYCOBACTIN

Introduction

Mycobactin is dispensed in 4 dram wide mouthed vials (2 mg). This quantity is sufficient for preparing a liter of Herrold's Egg Yolk Medium.

Preparation of Mycobactin

Add 4 ml of ethyl alcohol to the mycobactin. Mix until dissolved.

Preparation of Eggs:

(Note: Use fresh eggs--not more than 2 days old--from a flock that is not receiving antibiotics.)

With a brush, scrub eggs with water containing a detergent. Rinse with water, allow to dry and soak in 75% isopropyl alcohol for 30 minutes. Dry by inserting between two sterile towels. With sterile rat tooth forceps, crack one end of the egg shell, making about a 10 mm hole, and remove the egg white with the forceps and gravity. Make a hole larger and break yolk. Mix the egg yolk by twirling the forceps and remove the yolk sac. Pour the mixed egg yolk into media.

Formula for a Liter of Herrold's Egg Yolk Medium

Peptone	9.0 gm
NaCl	4.5 gm
Agar (Noble)	15.3 gm
Beef Extract (Difco)	2.7 gm
Glycerine	27.0 ml
Distilled water	870.0 ml
NaOH (4%)	about 4.1 ml

Mycobactin	contents of one vial dissolved in 4 ml ethyl alcohol
Egg Yolks	6
2% Malachite Green (oxalate form)(sterile)*	5.1 ml

Preparation of Herrold's Egg Yolk Medium

Measure ingredients 1-6 into a 2 liter flask. Dissolve by heating over a flame. Cool to about 60° C. Adjust pH to 7.5 by adding about 4.1 ml of 4% NaOH. Pour into 2 L. aspirator bottle to which a dispensing hose with small bell is attached. Add a magnetic stirring bar and, while mixing on a magnetic stirrer, add the mycobactin dissolved in 4 ml ethyl alcohol. The mycobactin should be added with a pipette, dropping it directly into the media rather than allowing it to come in contact with the inside surface of the aspirator. Autoclave at 121° C for 25 minutes. Cool to 56° C and aseptically add six sterile egg yolks and 5.1 ml of sterile 2% malachite green. Blend gently on magnetic stirrer and dispense into sterile tubes (8 to 10 ml per tube). Allow medium to harden in a slanted position. Check for sterility by incubating at 37° C for 48 hours.

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* Sterilize malachite green by autoclaving at 121° C for 15 minutes.

MIDDLEBROOK'S 7H-10 AGAR WITH MIDDLEBROOK OADC* ENRICHMENT

Ingredients	Per Liter
Middlebrook's 7H-10 Agar Base (Difco 0627)	20.0 gm
Distilled Water containing 5 ml Glycerol	900.0 ml
Middlebrook OADC Enrichment (Difco 0722)	100.0 ml

Method of Preparation

Mix and heat Middlebrook's 7H-10 agar base and water containing Glycerol until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing of flask for pouring plates. Sterilize in autoclave at 121° C for 15 to 20 minutes. Cool to 56° C and add Middlebrook OADC Enrichment. Mix well. Dispense in 20 x 125 screw cap tubes in 9 ml amounts and allow to solidify in slant position. For plates, pour medium into petri dishes in 25 ml amounts. Incubate at 37° C to check sterility.

Sodium pyruvate (Matheson 7264) may be added at the rate of 4.1 grams per 1000 ml of medium.

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* OADC - Oleic Acid, Bovine Albumin Fract V, Dextrose and Beef Catalase.

DUBOS BROTH WITH TWEEN 80 WITH DUBOS OLEIC ALBUMIN COMPLEX WITH INH*

Ingredients	Per Liter
Dubos Broth Base (Difco 0385)	6.5 gm
Distilled Water	890.0 ml
0.1% INH Solution (Isoniazid) (We use the Isoniazid from Squibb & Sons called Nydrazid 6375)	10.0 ml
Dubos Oleic Albumin Complex (Difco 0375)	100.0 ml

Method of Preparation

Mix Dubos Broth Base and water until in complete solution. Add the INH solution and mix thoroughly. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and mix well. Dispense aseptically into 20 x 125 screw cap tubes in 8 ml amounts. Incubate at 37° C to check sterility.

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* INH is abbreviation for Isoniazid - Isonicotinic acid hydrazid
INH concentration to be 10 micrograms per ml of media.

DUBOS BROTH WITH TWEEN 80 WITH DUBOS OLEIC ALBUMIN COMPLEX WITH TCH*

Ingredients	Per Liter
Dubos Broth Base (Difco 0385)	6.5 gm
Distilled Water	900.0 ml
Dubos Oleic Albumin Complex (Difco 0375)	100.0 ml
1% Solution of Thiophen-2-Carboxylic Acid-Hydrazid** (filter sterilized)	1.5 ml

Method of Preparation

Mix Dubos Broth Base and Water until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and the filter sterilized TCH solution. Mix well. Dispense aseptically into 20 x 125 screw cap tubes in 8 ml amounts. Incubate at 37° C to check sterility.

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* TCH is an abbreviation of Thiophen-2-Carboxylic Acid-Hydrazid - used at the rate of 15 micrograms per ml of media.

** Thiophen-2-Carboxylic Acid-Hydrazid can be obtained from the following sources:

Karl Roth, Herrenstrasse 26-28, Karlsruhe, Deutchland (Germany)

Aldrich Chemical Co., Inc., 2371 N. 30th St., Milwaukee, Wisconsin

DUBOS TWEEN ALBUMIN BROTH WITH NEOTETRAZOLIUM CHLORIDE*

Ingredients	Per Liter
Dubos Broth Base without Tween 80 (Difco 0435)	6.5 gm
Distilled Water	900.0 ml
Tween 80	0.5 ml
50% Glucose solution (filter sterilized)	10.0 ml
Dubos Oleic Albumin Complex (Difco 0375)	100.0 ml
1% Neotetrazolium Chloride Solution**	2.5 ml

Method of Preparation

Mix and warm Dubos Broth Base and water until in complete solution. Add Tween 80 to the hot medium and mix thoroughly. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to below 56° C. Add aseptically the sterile Glucose solution, Dubos Oleic Albumin Complex and the Neotetrazolium Chloride solution. Mix thoroughly. Dispense aseptically into 20 x 125 screw cap test tubes in 8 ml amounts. Incubate at 37° C to check sterility.

Glassware Instructions

Test tubes for this medium should be new or absolutely free from scratches so as not to hinder observation of media.

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* Neotetrazolium Chloride concentration to be 1 to 40,000.

** Autoclaved 15 minutes at 121° C.

DUBOS BROTH WITH TWEEN 80 WITH DUBOS OLEIC ALBUMIN COMPLEX WITH STREPTOMYCIN

Ingredients	Per Liter
Dubos Broth Base (Difco 0385)	6.5 gm
Distilled Water	890.0 ml
Streptomycin Stock Solution*	2.0 ml
Dubos Oleic Albumin Complex (Difco 0375)	100.0 ml

Method of Preparation

Mix Dubos Broth Base and water until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and Streptomycin solution and mix well. Dispense aseptically into 20 x 125 screw cap tubes in 8 ml amounts. Incubate at 37° C to check sterility.

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* Streptomycin Stock Solution - 1000 µg streptomycin per ml of distilled water. Filter sterilize.

DUBOS BROTH WITH TWEEN 80 WITH DUBOS OLEIC ALBUMIN COMPLEX WITH RIFAMPIN

Ingredients	Per Liter
Dubos Broth Base (Difco 0385)	6.5 gm
Distilled Water	890.0 ml
Rifampin Stock Solution*	0.5 ml
Dubos Oleic Albumin Complex (Difco 0375)	100.0 ml

Method of Preparation

Mix Dubos Broth Base and water until in complete solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to below 56° C. Add aseptically the Dubos Oleic Albumin Complex and Rifampin solution and mix well. Dispense aseptically into 20 x 125 mm screw cap tubes in 8 ml amounts. Incubate at 37° C to check sterility.

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* Stock Solution Preparation - 500 ug/ml

1. Dissolve 10 mg Rifampin in 1.0 ml of Dimethyl Sulfoxide (DMSO). This is solution "A" at 10,000 ug/ml.
2. Add 0.5 ml of solution "A" to 0.5 ml of Butterfield's Buffer. This is solution "B" at 5,000 ug/ml.
3. Add 1.0 ml of solution "B" to 9.0 ml of Butterfield's Buffer. This solution, at 500 ug/ml, is referred to above as "Rifampin Stock Solution."

MIDDLEBROOK'S 7H-9 BROTH*

Ingredients

Ammonium Sulfate	0.5	gm
1-glutamic Acid (Na Salt)	0.5	gm
Sodium Citrate	0.1	gm
Pyridoxine	0.001	gm
Biotin	0.0005	gm
Disodium Phosphate	2.5	gm
Mono Potassium Phosphate	1.0	gm
Ferric Ammonium Citrate	0.04	gm
Magnesium Sulfate	0.05	gm
Calcium Chloride	0.0005	gm
Zinc Sulfate	0.001	gm
Copper Sulfate	0.001	gm

Method of Preparation

To rehydrate the medium, dissolve 4.7 gm of the above mixture of ingredients in 900 ml distilled water containing 0.5 gm Tween 80. Distribute in 180 ml amounts and sterilize for 15 minutes at 15 lbs. pressure (121° C). To each 180 ml of sterile medium, add 20 ml of Middlebrook ADC enrichment and distribute 8 ml to each 20 x 125 screw cap test tube.

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* Media may be Purchased from Difco Laboratory, Detroit, Michigan.

MODIFIED P&B MEDIUM WITH 5% HORSE SERUM

Ingredients	Per Liter
L-Asparagine	5.0 gm
Potassium Phosphate Monobasic	5.0 gm
Potassium Sulfate	5.0 gm
Glycerol	20.0 ml
Distilled Water	930.0 ml
Magnesium Citrate	1.5 gm
Horse Serum (sterile)	50.0 ml

Method of Preparation

Dissolve L-Asparagine by heating in part of the water until clear. Dissolve each of the next two chemicals in small amounts of water separately. Add to asparagine mixture, add glycerol and rest of water. Mix thoroughly. Adjust pH to 7.0 with 10 Normal Sodium Hydroxide. Now add the 1.5 gm of Magnesium Citrate. Mix until in solution. Pour into aspirator bottle with attachment for aseptic dispensing. Sterilize in autoclave at 121° C for 20 minutes. Cool to 50° C and add aseptically the sterile Horse Serum. Dispense aseptically into 20 x 125 screw cap test tubes in 4 ml amounts. Incubate at 37° C to check sterility.

TRYPSIN

Preparation of a 2.5% Solution

Trypsin	25.0 gm
Distilled Water	1000.0 ml

Method of Preparation

Filter, sterilize, and package in 50 ml quantities in vaccine bottles.

PAPAIN - CYSTEINE HYDROCHLORIDE SOLUTION

Ingredients	Per Liter
Papain	50.0 gm
Cysteine Hydrochloride	0.6 gm
Distilled Water	1000.0 ml

Method

Heat solution to 70° C after mixing. Filter sterilize through Seitz or Millipore filter. Dispense aseptically into screw cap dilution bottles in 100 ml amounts. Refrigerate immediately for storage.

PENTANE

A working solution of Pentane is obtained by transferring directly from the manufacturers container to sterile screw cap test tubes. The screw caps should be equipped with Teflon liners. Rubber liners are attacked by Pentane and organic compounds of undetermined composition are released. Their effect on the viability of mycobacteria has not been determined.

Care should be taken to avoid introducing bacterial contamination in the Pentane or on the equipment used to handle it.

CAUTION: Pentane is highly volatile - exposure to sparks or flame must be avoided.

BENZALKONIUM CHLORIDE
(Zephiran)

A 0.2% working solution is prepared by mixing 1.2 ml of 17.0% Zephiran with 98.8 ml of sterile distilled water. A 0.3% working solution is prepared by mixing 1.8 ml of 17.0% Zephiran with 98.2 ml of sterile water. Individual aliquots may be distributed to screw cap vials for use with individual cases.

Zephiran is manufactured by:

Winthrop Laboratories
New York, New York

NIACIN TEST REAGENTS

Cyanogen Bromide Solution

Cyanogen Bromide	10.0 gm
Distilled Water	90.0 ml

Ethyl Aniline Solution

Aniline	4.0 ml
95% Ethyl Alcohol	96.0 ml

CAUTION: Cyanogen bromide solution should be prepared and used in a fume hood. Both solutions should be stored in brown bottles. They should be kept no longer than four weeks before replacement with fresh solutions.

REAGENTS FOR ZIEHL-NEELSEN ACID-FAST STAIN

Stock Saturated Alcoholic Basic Fuchsin Solution

95% Ethyl Alcohol	100.0 ml
Basic Fuchsin	3.0 gm

Working Solution Basic Fuchsin

Saturated Alcoholic Basic Fuchsin	10.0 ml
5% Phenol (Analytical Grade)	90.0 ml

Acid Alcohol

Hydrochloric Acid (conc.)	3.2 ml
95% Ethyl Alcohol	97.0 ml

Brilliant Green Counterstain

Brilliant Green	1.0 gm
.01% NaOH	100.0 ml

PROCEDURE FOR PERFORMING THE ZIEHL-NEELSEN ACID-FAST STAIN

1. Fix the smear by heating gently.

2. Stain the fixed smear for three minutes in steaming Ziehl's carbol fuchsin. (A strip of filter paper laid on the smear tends to conserve reagent and impart uniform distribution of stain.)

3. Rinse in distilled water.

4. Decolorize in 95% ethyl alcohol, containing 3% by volume of concentrated hydrochloric acid, until only a suggestion of pink color remains. 2-3 minutes.

5. Wash in distilled water.

6. Counterstain for three minutes with alkaline brilliant green.

7. Wash in distilled water and dry; read with oil-immersion lens of microscope.

BUTTERFIELD'S BUFFER

Stock Solution

Potassium Phosphate Monobasic KH_2PO_4	34.0 gm
Distilled Water	500.0 ml

Adjust pH to 7.2 with 1 N NaOH (requires approximately 180 ml 1 N NaOH).

Add water QS to 1000 ml.

Diluent

Add 1.25 ml of above to each 1000 ml of buffer needed.

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